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Priby Council

MEDICAL RESEARCH COUNCIL

THE REACTION OF CULTURE MEDIA

BY

S. R. DOUGLAS, J. W. H. EYRE, P. P. LAIDLAW
and C. G. L. WOLF

Revised by P. P. LAIDLAW

LONDON
PUBLISHED BY HIS MAJESTY'S STATIONERY OFFICE
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THE REACTION OF CULTURE MEDIA

By S. R. Douglas, M.R.C.S., F.R.S.; J. W. H. Eyre, M.D.;
P. P. Laidlaw, B.Ch.; C. G. L. Wolf, M.D.;
revised by P. P. Laidlaw, B.Ch.

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Introduction.

Accurate knowledge of the reaction of culture media is important from two different points of view. First it has been shown that the initial reaction of a culture medium should be set with accuracy in order to secure good growth; secondly the final reaction of a medium after maximum growth has occurred frequently yields information of value.

Many observers have pointed out that although some bacteria will grow in media having a wide range of reaction, there are certain reactions at which optimum growth takes place. On the other hand, there are certain bacteria which require a more limited range, so that to obtain any appreciable growth the reaction of the medium employed must be accurately defined within these limits.

Until a few years ago, the reaction of a medium was expressed in terms of the amount of a standard normal or decinormal acid which would be necessary to bring the medium to neutrality, using litmus paper or phenolphthalein solution as an indicator. This method served a very useful purpose in adjusting media of similar composition to a more or less arbitrary standard which trial had shown to be generally suitable for growth. Later experience has demonstrated that these titration tests do not give the true reaction when carried out in media of different compositions, and, what is more disadvantageous, tests carried out by individual workers, under what are

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presumed to be identical conditions, lead to widely divergent results. This is shown in a table given by Clark in which eight careful observers, using the same apparatus and the same medium, obtained results varying between 0·7 c.cm. and 5·1 c.cm. of a $\frac{N}{40}$ NaOH solution in titrating a 5 per cent. peptone solution. It is clear that the reactions of these neutralized peptones cannot have been the same, and that errors of this size may be serious.

If under these carefully controlled conditions the variations were so great, it can be safely assumed that workers in different laboratories would encounter even wider discrepancies when attempting a similar series of experiments.

The causes of the errors in titration are partly due to the personal equation of the manipulator, his colour sense, &c., but for the most part to the fact that the end point is not sharp. Near the end point of the titration there is a zone where the addition of acid or alkali does not produce a striking change in the colour of the indicator (see Buffers, p. 7).

The true reaction of any fluid is determined by the amount of hydrogen or hydroxyl ions which are present in it. For theoretical and practical reasons which are not for the moment important, the reaction of a fluid is therefore expressed by the concentration of hydrogen ions which it contains. Using this standard to check the results of the older titration method, an example of wide departure from what was expected may be quoted. A 'neutral' plain broth was prepared, using the titration method to determine its reaction. It was found to have a pH of 8·4. This, as will be shown later, is a difference between $1{\cdot}0 \times 10^{-7}$ (= neutrality) and $0{\cdot}04 \times 10^{-7}$, or a difference in hydrogen ion concentration of 25 to 1. It is evident that errors of this magnitude may readily give rise to difficulty, or mistakes, when the bacteriologist is dealing with organisms which only grow well within somewhat narrow limits of reaction. It would appear, therefore, that the determination of the true reaction of a medium should be adopted in place of the titration figure now in common use, provided that this can be done simply and expeditiously.

In accurate determinations of hydrogen ion concentrations the hydrogen electrode is used. The principle involved in this determination is that if a solution containing a certain concentration of hydrogen ions is in contact with a metal saturated with hydrogen, a difference of electrical potential is set up due to the transfer of hydrogen ions from the place of saturation (the electrode) to the place where the hydrogen ions are in lesser concentration (the solution). Depending on the concentration of hydrogen ions in the solution, this difference will vary, and can be measured. From this measurement the concentration of hydrogen ions in the solution can be readily calculated. The details of the method, in so far as bacteriological media are concerned, will be found in papers by Walpole and by Barendrecht.

In ordinary laboratory practice very accurate results may be obtained by a colorimetric method, which is comparatively simple in

its application. This is the method advocated and described later (pp. 8–16). Nevertheless it must be emphasized that the determination of the hydrogen ion concentration by the hydrogen electrode is the final court of appeal.

The simple considerations on which the true acidity of a solution are based may be stated as follows :

The acidity is expressed in terms of the weight of free hydrogen ions present in a litre of solution. This is entirely analogous to the ordinary expression of the strength of solutions as ' normal ' or ' decinormal ', *but only* when the following considerations are taken into account.

In a normal solution of hydrochloric acid one has 36·5 grammes of the acid in solution, of which 35·5 grammes consist of chlorine and one gramme is hydrogen. In the case of hydrogen ion concentration the anions (chlorine in the case of hydrochloric acid) are neglected, and the kation, hydrogen only, is referred to.

Hence, in a normal solution of hydrochloric acid there is approx. 1 gramme of hydrogen per 1,000 c.cm. The concentration is therefore 1/1000. As the concentrations of hydrogen ions dealt with in biology are very much smaller than this, it was found to be more convenient to express the normality in terms of negative powers of ten. Thus in dealing with 0·1 normal solution, the expression is 1×10^{-1} ; 0·01 normal $= 1 \times 10^{-2}$; 0·001 normal $= 1 \times 10^{-3}$, &c.

In a solution of hydrochloric acid, the dissociation of the hydrogen and chlorine into the respective H and Cl ions is nearly complete. If it were complete, a 0·001 normal solution would have a hydrogen ion concentration of 1×10^{-3}. This is not strictly the case, for a certain amount of the acid is not dissociated, and therefore the amount of hydrogen ions is less than 1×10^{-3}. Investigation has shown that the real concentration is $0{\cdot}97 \times 10^{-3}$, because only 97 per cent. of the acid is completely dissociated.

The difference in the case of hydrochloric or any other ' strong ' acid is not great, but in dealing with organic acids, such as are frequently encountered in biological work, a much greater difference will be found. This is due to the fact that these acids dissociate much less completely than hydrochloric acid. For example, in the case of 0·001 normal acetic acid, which would have a hydrogen ion concentration of $0{\cdot}97 \times 10^{-3}$ if it were as completely dissociated as hydrochloric acid, experiments have shown that the real concentration is only $0{\cdot}136 \times 10^{-3}$, that is to say, acetic acid has only $\frac{0{\cdot}136}{0{\cdot}97}$ or approximately one-seventh of the amount of hydrogen ions contained in a similar solution of hydrochloric acid.

The method of expressing the hydrogen ion concentration directly has proved cumbrous and inconvenient, for two numbers are involved in the expression, viz. the actual amount and the minus power to which this is raised. It is not easy to use this expression in constructing curves except by keeping the power constant, and then the figures become so great that a very large graph is necessary for

their exposition. The second difficulty is that the hydrogen electrode method of determining the reaction, on which all these data are based, does not give the results directly in this form. On this account Sörensen proposed designating both figures in terms of a negative logarithm, and the custom has been generally followed. The minus sign is understood, but is generally omitted. Hence 1×10^{-5} becomes 5·0; $1 \times 10^{-6} = 6{\cdot}0$, &c. This value is known as the 'hydrogen exponent' and is usually written pH.

The only difficulty for the beginner is in translating the actual hydrogen ion concentration into pH when the amount is a fraction. For example, it is quite easy to understand that 1×10^{-5} is equivalent to pH 5·0, but the process of transforming $2{\cdot}0 \times 10^{-5}$ into pH is not so simple. The method of conversion is as follows:

$$1{\cdot}00 \times 10^{-5} \text{ or } \mathrm{pH} = 5{\cdot}00$$
$$2{\cdot}00 \times 10^{-5} \text{ or } \mathrm{pH} = 4{\cdot}7,$$

for the logarithm of 2·0 = 0·3. This added to $-5{\cdot}00 = -4{\cdot}70$, or, the minus sign being always understood, = 4·7.

To take a practical example. The hydrogen ion concentration of 0·001 normal acetic acid is $1{\cdot}36 \times 10^{-4}$. The logarithm of 1·36 = 0·133. Hence the pH of this solution is $0{\cdot}133 - 4{\cdot}00 = 3{\cdot}867$.

The following table, taken from the paper of Hurwitz, Meyer, and Ostenberg, gives the relation between some constantly recurring hydrogen ion exponents and the actual concentration.

pH.	*Hydrogen ion concentration.*	*pH.*	*Hydrogen ion concentration.*
6·4	$4{\cdot}0 \times 10^{-7}$	7·5	$0{\cdot}32 \times 10^{-7}$
6·6	$2{\cdot}5 \times 10^{-7}$	7·6	$0{\cdot}25 \times 10^{-7}$
6·8	$1{\cdot}6 \times 10^{-7}$	7·7	$0{\cdot}20 \times 10^{-7}$
7·0	$1{\cdot}0 \times 10^{-7}$	7·8	$0{\cdot}16 \times 10^{-7}$
7·1	$0{\cdot}8 \times 10^{-7}$	8·0	$0{\cdot}10 \times 10^{-7}$
7·2	$0{\cdot}63 \times 10^{-7}$	8·2	$0{\cdot}063 \times 10^{-7}$
7·3	$0{\cdot}5 \times 10^{-7}$	8·4	$0{\cdot}040 \times 10^{-7}$
7·4	$0{\cdot}4 \times 10^{-7}$		

Two features must be noted when dealing with such minus logarithms. One is that the actual amount of hydrogen ions increases as the pH decreases. That is to say, pH = 7·0 represents a more acid solution than pH = 8·0. The second point is that as these are logarithms the amounts represented are logarithmic and not simple quantities; thus, as will be seen by a reference to the above table, a pH of 7·7 represents a solution containing twice the number of hydrogen ions contained in a solution with pH = 8·0. Similarly, a pH of 3·0 indicates that the solution has ten times as many hydrogen ions as a solution of pH = 4·0.

Neutrality.

It has been ascertained by experiment that the total concentration of hydrogen ions existing in the purest water at 22° C. $= 1 \times 10^{-7}$. Hence the hydrogen exponent of water is 7·00. This must

represent neutrality, because in this liquid the hydrogen and hydroxyl ions are equally balanced, any dissociation of water which takes place gives H^- and OH^+ in equal quantity. The optimum growth of most bacteria takes place in this region. The attempts which have been made to fix a neutral point in bacteriological media by means of titration have usually resulted in a fluid which is on the alkaline side of neutrality. Whether this is more advantageous for growth than strict neutrality has not been determined. We do know, however, that the reaction of the blood fluids is in the region of $pH = 7{\cdot}5$.

All bacteria, in fact all forms of living matter, produce acids in the course of growth. With animals, lactic acid is one of the principal products. Bacteria also produce this acid, and, in addition, formic, acetic, propionic, butyric, and other higher acids are formed. These lead to the acidification of the medium. This is a familiar experience with *B. coli*, one of the characteristics of which is to produce so much acid in a medium containing certain carbohydrates that neutral red changes colour.

The accumulation of acids is one of the main causes of the cessation of growth. It can be shown that not only do bacteria stop growing when they have produced sufficient acids to give rise to a certain hydrogen ion concentration, but that a medium originally adjusted to such a reaction absolutely inhibits growth. The same medium made slightly more alkaline will permit growth.

The question of acid production and its effect on the growth of bacteria is important, for one of the factors in securing an abundant growth is to have the initial reaction of the medium at the proper level, and a second is to prevent the acids produced from effecting a significant change in the reaction of the fluid.

Regulators or Buffers.

An abrupt change in the reaction of the medium when acids are developed during the growth of an organism is prevented by the presence of certain compounds which have the property of taking up hydrogen ions. This property is possessed by proteins, amino acids, and certain salts such as the soluble phosphates, carbonates, borates, &c. All these compounds are called ' regulators ' or ' buffers '. They take up hydrogen ions so that the addition of moderate quantities of acids makes a comparatively small change in the reaction of the fluid.

The so-called ' buffer effect ' has been experienced by all who have titrated bacteriological media. The usual experience is that ' a sharp end point ' is not obtained. The colour changes gradually as alkali or acid is added, and the full and pure colour of the indicator is not given until what is apparently a large excess of reagent has been added. This is quite different from the sharp change which is observed when a strong alkali and acid are titrated against one another, using the same indicator.

INDICATORS.

The gradual colour change in the indicator when used in organic media is due partly to the circumstance that an indicator has a considerable range of reaction through which it changes colour ; thus phenolphthalein commences to acquire a pink colour at pH = 8·00, but does not develop its fullest colour until pH = 10·0. Various indicators change at different ranges : methyl orange, for example, changes between pH = 3·00 and pH = 4·7. Intermediate between these we have a number of indicators whose range of change has been worked out. It is due to Clark, and those who have worked with him, that the following series of indicators has been investigated which have colour changes between pH = 1·0 and pH = 10·0 :

List of Indicators and their Range.

Indicator.	*Range.*
Thymol sulphonephthalein, acid range (Thymol Blue)	1·2–2·8
Tetrabromphenol sulphonephthalein (Bromphenol Blue)	2·8–4·6
Orthocarboxybenzene azodimethylaniline (Methyl Red)	4·4–6·0
Dibromcresol sulphonephthalein (Bromcresol Purple)	5·2–6·8
Dibromthymol sulphonephthalein (Bromthymol Blue)	6·0–7·6
Phenol sulphonephthalein (Phenol Red)	6·8–8·4
Orthocresol sulphonephthalein (Cresol Red)	7·2–8·8
Thymol sulphonephthalein, alkaline range (Thymol Blue)	8·0–9·6

The description of these indicators and the method of using them will be found in Clark's papers referred to in the bibliography at the end of this report.

One of the most useful is phenol sulphonephthalein, or, as it is usually called, ' phenol red '. The colour changes are best appreciated between pH = 7·2 and pH = 8·0. It is in this region that the most useful reactions for bacteriological media occur. Within the range pH = 7·2 to pH = 8·0 the tint of a clear and colourless solution to which a little phenol red has been added indicates the hydrogen ion concentration of that solution, and hence its true reaction. While phenol red is suitable for this range of reactions, other indicators must be used when other regions of acidity are investigated.

The initial reaction of a bacteriological medium may be determined and controlled, in terms of hydrogen ions, by adopting the following procedure. A solution of definite and known hydrogen ion concentration is prepared, and to this a small amount of a suitable indicator is added. The tint of this forms the standard to which the medium must be brought by titration. A precisely similar proportion of indicator is added to a small sample of the medium and the tint compared with that of the standard, care being taken that similar thicknesses of solution are viewed in both cases. Acid or

alkali is added from a burette until the colours of test solution and standard match. The yellow-brown colour of the original untreated medium is readily and accurately compensated for, in making the colour match, by interposing a tube of untreated medium between the standard tube and the source of light.[1]

With a series of solutions of known hydrogen ion concentration and a suitable series of indicators any initial reaction may be imparted to a medium within the range pH = 1·0 to pH = 10·0.

Further, the final hydrogen ion concentration resulting from bacterial growth may be rapidly found by merely adding indicator and comparing the tint with the series of standards. The whole process is simple, rapid, and accurate when the standards are available.

The Preparation of Solutions of definite known Hydrogen Ion Concentration.

For range of reaction between pH = 6·5 and pH = 8·0 a suitable series of standard solutions may be made from a solution of disodium hydrogen phosphate (Na_2HPO_4)—the 'sodium phosphate' of the British pharmacopoeia—and acid potassium phosphate (KH_2PO_4).

As the proportion of the potassium dihydrogen phosphate (KH_2PO_4) increases, the hydrogen ion concentration of the solution increases owing to partial hydrolysis of the salt.

The method of employing phosphate solutions of a definite hydrogen ion concentration and phenol red as an indicator, as standards for bacteriological work, was introduced by Hurwitz, Meyer, and Ostenberg on the basis of the previous work of Levy, Rowntree, and Marriott, who had determined the reserve alkali of the blood in this way. Hurwitz and his co-workers compensated for the colour of the medium by placing tubes of media in a line with the standard solution. In this way a very perfect compensation is easily accomplished. It is the same method which Walpole had previously employed in a different way. The standard solutions of Hurwitz, Meyer, and Ostenberg have been since employed by Cole and Onslow and by Clark. Cole and Onslow use two standard solutions instead of one, and Clark has developed media based on the reaction between potassium phthalate and acid potassium phosphate and hydrochloric acid or sodium hydroxide. With these reagents standard hydrogen ion solutions may be prepared ranging from pH = 2·11 to pH = 8·02.

The solutions necessary for preparing one set of standards of definite hydrogen ion concentration (pH 6·24 to pH 8·04) are :

1. A 1/15 molecular solution of acid potassium phosphate KH_2PO_4.
2. A 1/15 molecular solution of disodium phosphate $Na_2HPO_4\ 2H_2O$.
3. A 0·02 per cent. solution of phenol red.

The following are the directions for preparing these solutions :

1. A pure acid potassium phosphate is prepared by recrystallizing

[1] There are certain salt and protein errors which occur with some indicators which cannot be disregarded when small differences are being noted. A discussion of these errors will be found in a paper by Clark and Lubs, *J. Bact.*, 1917, **2**, 123.

the salt two or three times from distilled water. It is dried first between filter paper and finally in a water oven at 100° C.

Of this salt, 9·078 grammes are weighed out and dissolved in about 500 c.cm. of distilled water contained in a litre flask; 45·5 c.cm. of the indicator solution are added and the volume made up to 1,000 c.cm. with distilled water.

2. The dihydrate of disodium phosphate has been found to be the most suitable for preparing a standard solution. It is made as follows: A quantity of the crystalline sodium phosphate of the pharmacopoeia, which should be of the purest quality, is placed in a flat dish in a layer not more than 1–2 cm. deep. It is covered with paper to protect from dust and allowed to stand for about two weeks at room temperature. The room should be dry; for when the atmosphere is very humid, the salt may not lose the necessary quantity of water. The salt should be turned over with a spatula every second day, and any of the larger lumps crushed. At the end of two weeks the salt is transformed into a snow-white granular substance having the composition Na_2HPO_4, $2H_2O$. A check may be made of the loss of weight during the last few days by weighing out about a hundred grammes with an accuracy of 0·1 gramme and weighing again in the course of a couple of days. The two weights should agree to 0·1 gramme. It is advisable to have the water content of the final product checked by analysis. This may be done by determining the loss in weight when the salt is dried to constant weight at 98° C. in a water oven. The loss should equal 20·2 per cent. of the weight of the dihydrate. A 1 per cent. solution should give a red colour with a few drops of phenolphthaleine. Of the dihydrate so prepared, 11·876 grammes are weighed out and dissolved in about 500 c.cm. of distilled water contained in a litre flask. 45·5 c.cm. of the indicator solution are added and the volume made up to 1,000 c.cm.

3. The 0·02 per cent. solution of phenol red is prepared by dissolving 0·1 gramme of phenol sulphonephthalein in about 100 c.cm. of distilled water to which 10 c.cm. of N/10 sodium hydroxide solution have been added. The solution may be hastened by gentle heating. About 300 c.cm. of water are added, and the alkaline solution of the indicator is exactly neutralized with 10 c.cm. of N/10 hydrochloric acid. The volume is then made up to 500 c.cm.

The Preparation of Standards.

The standard phosphate solutions are based upon the following data taken from Sörensen's table. The mixtures have the corresponding hydrogen ion exponent set against them.

c.cm. N/15 Na^2HPO^4.	*c.cm. N/15 KH^2PO^4.*	*—log. (H) or pH.*
2	8	6·24
3	7	6·47
4	6	6·64
5	5	6·81
6	4	6·98
7	3	7·17
8	2	7·38
9	1	7·73
9·5	0·5	8·04

From this table by means of interpolation the following table of mixtures has been constructed. These figures have been checked by actual determinations with the hydrogen electrode, and are found sufficiently correct for practical purposes.

c.cm. of 1/15 molecular Na_2HPO_4 solution.	*Diluted with 1/15 molecular KH_2PO_4 solution to*	*pH.*
38·0	100	6·6
43·5	100	6·7
49·5	100	6·8
55·5	100	6·9
61·0	100	7·0
66·5	100	7·1
72·0	100	7·2
76·5	100	7·3
80·5	100	7·4
84·0	100	7·5
86·5	100	7·6
89·0	100	7·7
91·3	100	7·8
93·0	100	7·9
94·5	100	8·0

The apparatus necessary for preparing the standard tubes of definite hydrogen ion concentration are :

1. Standard burette, 50 c.cm. graduated to 1/10ths.
2. Standard 100 c.cm. flasks.
3. Tubes to contain the standard solutions.

These are made from ' cordite ' tubes.[1] They are thoroughly cleaned by boiling with distilled water, rinsed out several times with water, and dried in the water oven. They are then drawn out in the blow-pipe flame about 8 cm. from the sealed end so that a constriction is formed about 3 mm. in diameter through which they can subsequently be filled.

4. Small capillary funnel made from a test-tube by drawing out a neck less than 3 mm. in diameter and about 15 cm. long.

The method of making the mixtures is as follows :

The volume of the disodium phosphate solution is measured into a carefully cleaned 100 c.cm. graduated flask from the burette, and the volume made up exactly to 100 c.cm. with the acid potassium phosphate. The mixture so obtained will have the corresponding hydrogen exponent.

The ' cordite ' tubes are then filled with this solution, using the small capillary funnel. It is advisable to place a few small crystals of thymol in the tubes to prevent any bacterial growth. The tubes are numbered and the proper pH indicated upon them, and are sealed in the blow-pipe and are ready for use. They should be kept in the dark when not in use.

[1] ' Cordite tubes ' are specially selected tubes of hard white glass which have a uniform thickness of wall and external diameter. They are used in certain colour reactions in the routine testing of cordite. Test-tubes of good quality may also be used, provided that a careful selection is made, so that they are all of the same measurements. The cordite tubes may be obtained from Messrs. Baird and Tatlock, Cross Street, Hatton Garden, London, E.C.

As the dihydrate of sodium phosphate is somewhat difficult to prepare, and the dihydrogen potassium phosphate crystallizes with ease, an alternative method is given for preparing solutions of definite hydrogen ion concentration from the latter salt.

The solutions necessary are:

1. A 1/5 normal solution of dihydrogen potassium phosphate containing phenol red. This is made by dissolving 13·616 grammes of recrystallized KH_2PO_4 in about 300 c.cm. of distilled water freshly boiled to remove dissolved carbon dioxide and then cooled. To the solution is added 90·9 c.cm. of a 0·02 per cent. solution of phenol red. The mixture is then made up to 500 c.cm.

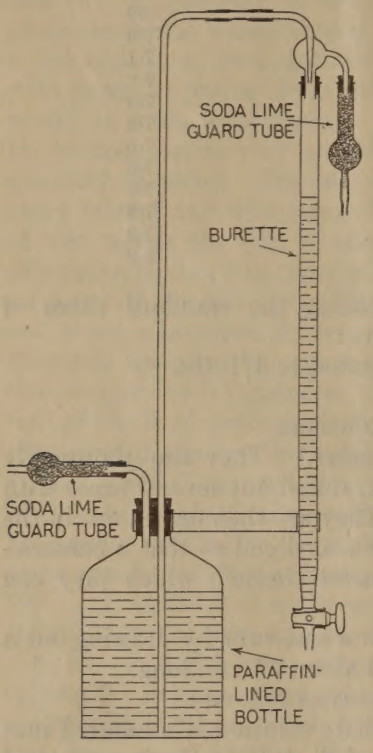

FIG. 1. Diagrammatic Section of Apparatus.[1]

2. A N/10 solution of sodium hydroxide free from carbonates.

The standard solution of caustic soda may be made from good quality stick soda in the following manner. Weigh out 100 grammes of soda sticks and dissolve in 100 c.cm. of distilled water, using a hard glass flask as a container. Cover the mouth of the flask with tin foil and set aside to stand for, at least, twenty-four hours. In this very strong solution the sodium carbonate separates out almost quantitatively and collects on the bottom and sides of the flask. The weighing need not be exact and it is preferable to use a freshly opened bottle of stick soda as soda sticks which have been exposed to the air have a higher proportion of carbonate.

It is sometimes possible to siphon off perfectly clear supernatant fluid free from carbonate, but if this should prove to be impracticable the solution must be filtered. Filtration is most readily effected through a layer of asbestos in a Gooch crucible.

The clear filtrate or supernatant fluid should be more than ten times normal and must be diluted with distilled water which has been boiled thoroughly to expel dissolved carbon dioxide and then cooled with minimum exposure to the air. Ten cubic centimetres of the strong solution are diluted to one litre. A sample of this first dilution is withdrawn and titrated with standard acid. The result of this titration shows the further dilution necessary to bring the solution to decinormality. The requisite dilution is made, again with distilled water free from CO_2 and the final product is at once transferred to a

[1] This apparatus can be obtained from Messrs. Baird and Tatlock, Ltd.

paraffin-lined bottle, to which a burette and soda-lime guard tubes have been attached (see Fig. 1). All joints are sealed with paraffin wax so that air is not allowed to come in contact with the solution unless it first passes through the soda-lime tubes which absorb the atmospheric carbon dioxide. The final product is next accurately standardized against acid of known normality or against weighed amounts of pure acid potassium phthalate (Mol. Wgt. 204·14). It is probable that the final solution will not prove to be precisely decinormal, but it is better to note the error and employ a correcting factor in all calculations rather than expose the solution to the air in an endeavour to achieve absolute accuracy.

From these two solutions the following mixtures are made up :

N/5 KH_2PO_4.	*N/10 NaOH.*	*Water to*	*pH.*
25	17·80	100	6·6
25	23·65	100	6·8
25	29·63	100	7·0
25	35·00	100	7·2
25	39·50	100	7·4
25	42·80	100	7·6
25	45·20	100	7·8
25	46·80	100	8·0

This is done by measuring out 25 c.cm. of the phosphate solution with a pipette into a standard 100 c.cm. flask. The requisite amount of N/10 sodium hydroxide solution as given in the table is added from the burette and the volume of the mixture made up to 100 c.cm. with distilled water.

The standard phosphate mixtures made up as described cover a limited range of hydrogen ion concentrations round the neutral point, and for the initial reaction of culture media, in the great majority of instances, this series of standards will suffice. There are, however, occasions on which it is desired to set the initial reaction at some other definite level outside the limits of the phosphate series ; and after maximal growth of an organism has occurred the final reaction may be widely removed from the phosphate zone.

By adopting a similar technique but employing other sets of salts in the place of the phosphates, and using different indicators, several series of standards may be made which, taken together, will cover a wide range of hydrogen ion concentrations. Thus, acetic-acid-sodium-acetate mixtures (1/5 Mol.) may be made to cover the zone pH 3 to pH 6 (Walpole). Acid potassium phthalate, with hydrochloric acid, or with caustic soda, can be used to give two series of standards which together cover a zone from pH 2·2 to pH 6·2 (Clark and Lubs). These mixtures may be employed with an indicator which gives a useful colour change round about the desired reaction. For details with regard to these and other similar mixtures, the purification of the salts, and the best indicators to use, the reader is referred to Clark's book, *The Determination of Hydrogen Ions*, where full information is available.

It should be thoroughly appreciated that the task of making up sets of tubes containing standard solutions of definite hydrogen ion

concentration is one in which scrupulous care must be observed, and only the purest chemicals must be used. It is a laborious process and though simple for a chemist it consumes a great deal of time.

The British Drug Houses, Ltd. will supply stock solutions of reagents of analytical purity, at the correct strength for the manufacture of standard mixtures.

It has been shown that the labour of making all these standard mixtures may be avoided and yet a reasonably accurate result secured by a different method, which was first introduced by Barnett and Chapman for the indicator phenol red. Gillespie extended the method to other suitable indicators, and placed it on a more satisfactory and solid basis. By the use of this method and employing the indicators listed on p. 8, Gillespie showed that it was possible to make up colour standards corresponding to hydrogen ion concentrations within the range of pH 3·1 to pH 9·7 with very little trouble and great rapidity.

In a solution of a given hydrogen ion concentration containing indicator there will be a certain definite proportion of indicator in the acid form and a complementary proportion in the alkaline form. Light traversing such a mixture will be affected by both forms independently, and the tint of the emergent beam will depend on the proportion of acid to alkaline indicator. There should be no difference whether the two forms of indicator are present in one solution or if they are in separate containers. These considerations, which involve certain assumptions, suggest that the tint of a solution of definite hydrogen ion concentration containing indicator can be accurately matched by two separate layers of fluid, where one layer contains a certain proportion of indicator in the acid form and the other a complementary proportion of indicator in the alkaline form. This has been shown to be the case for specific indicators. In the new method, therefore, each standard consists of twin tubes, one containing indicator with excess of acid, the other indicator with excess of alkali. In use they are so arranged that light passes directly through both tubes, and thus the emergent light is the resultant of the influence of a known proportion of acid and alkaline indicator.

Gillespie has determined the various proportions of acid and alkaline indicators which, viewed in combination, yield tints that correspond to definite pH values, and in addition to making careful theoretical calculations he has tested out the method against Clark and Lubs standard mixtures. He finds the results accurate to within about 0·1 pH and concludes that it yields a good approximation to the truth. It cannot replace the hydrogen electrode, and it is less accurate than good standards of the phosphate type which have been described in detail; but it is so convenient for occasional use, relatively simple to manipulate, and gives results so near the truth, that it is recommended for general use. At the same time it is advised that whenever this method is used, the fact should be stated, as it cannot be regarded as precisely accurate.

Apparatus required:

1. Cordite test-tubes of approximately equal bore marked at the 5 c.cm. and 10 c.cm. levels.
2. Dropping pipettes.
3. Solutions of indicators as listed on p. 8. Phenol red, cresol red, methyl red, 0·02 per cent.; thymol blue, bromphenol blue, bromcresol purple, bromthymol blue, 0·04 per cent.; all in alcoholic solution.
4. Solutions of hydrochloric acid *approx.* 0·05 *Mol.*; acid potassium phosphate *approx.* 2 *per cent.*; and caustic soda *approx.* 0·2 *per cent.*
5. Distilled water.

Two rows of nine tubes are placed in a rack and into these the solution of the selected indicator is distributed by the drop method. In the front row of nine tubes the drops increase regularly from one to nine; in the back row the drops decrease from nine to one; so that the sum total of drops in any pair of tubes will be ten. To each tube in the front a drop of alkali is added (two drops in the case of thymol blue); and to each of the tubes in the back row sufficient acid is added to develop the full acid colour of the indicator employed. The tubes are then filled up to the 5 c.cm. mark with water.[1]

This duplex series of tubes, placed in a comparator rack so that light passes directly through both acid and alkaline tubes, gives a gradation of colour change very similar to that of a single series of standard salt solutions with added indicator. They are used in pairs, in the same manner as the phosphate standards. The only difference that need be noted is that the comparator rack employed should be capable of holding sets of tubes three deep, in order to allow of the interposition of control tubes of medium behind the twin standards and water blanks behind the tube of medium the reaction of which is to be determined. In this way correction for the natural colour of the medium is made. Ten drops of indicator are added to the unknown (see later).

There are certain limitations in this method which depend on the properties of indicators. Some indicators such as phenolphthalein cannot be used in this way, and those that are employed must be used with judgement. Thymol blue, for example, has two separate ranges of colour change, an alkaline range from pH 8 to pH 9·6, and an acid range from pH 1·2 to pH 2·8. Clearly the acid employed in the acid series of tubes is important, for if large excess of acid be added to this indicator the resultant colour will be of the kind associated with the acid range pH 1·2, &c., and quite unsuitable for the alkaline range pH 8, &c. To obviate this source of error acid potassium phosphate is used for the acid tubes in making up the low range standards for this indicator and also in the case of cresol red. On the other hand when bromphenol blue is employed one cubic centi-

[1] The 10 c.cm. mark is used with twice the number of drops given in the table, as this is the most convenient method of securing the half drops shown at either end of the drop scale.

metre of 0·05 *Mol.* hydrochloric acid is used in the acid tubes in order to ensure the full production of the acid tint.

Gillespie's table of pH values corresponding to various drop-ratios.

Drop-ratio. Alk : Acid.	*Brom-phenol blue.*	*Methyl red.*	*Brom-cresol purple.*	*Brom-thymol blue.*	*Phenol red.*	*Cresol red.*	*Thymol blue.*
1 : 9	3·1	4·05	5·3	6·15	6·75	7·15	7·85
1·5 : 8·5	3·3	4·25	5·5	6·35	6·95	7·35	8·05
2 : 8	3·5	4·4	5·7	6·5	7·1	7·5	8·2
3 : 7	3·7	4·6	5·9	6·7	7·3	7·7	8·4
4 : 6	3·9	4·8	6·1	6·9	7·5	7·9	8·6
5 : 5	4·1	5·0	6·3	7·1	7·7	8·1	8·8
6 : 4	4·3	5·2	6·5	7·3	7·9	8·3	9·0
7 : 3	4·5	5·4	6·7	7·5	8·1	8·5	9·2
8 : 2	4·7	5·6	6·9	7·7	8·3	8·7	9·4
8·5 : 1·5	4·8	5·75	7·0	7·85	8·45	8·85	9·55
9 : 1	5·0	5·95	7·2	8·05	8·65	9·05	9·75
Produce acid colour with	1 c.cm. of 0·05 M. HCl.	1 drop of 0·05 M. HCl.	1 drop of 0·05 M. HCl.	1 drop of 0·05 M. HCl.	1 drop of 0·05 M. HCl.	1 drop of 2% KH_2PO_4.	1 drop of 2% KH_2PO_4.

Twin standards of this type can be sealed with paraffined corks and they can then be kept for a time, but they are so quickly and easily prepared, once the apparatus is assembled, that new standards should be made up at frequent intervals.

The Adjustment of the Reaction of Media

It is usually desired to prepare a medium which has a reaction in the neighbourhood of pH = 7·0 to pH = 7·5. Whatever the reaction of the medium may be before it is adjusted, it is useful to add so much alkali in the form of a normal sodium hydroxide or a normal sodium carbonate solution that a drop of the medium on a piece of coralline paper gives a faint rose tint.[1] A medium which is made alkaline to this extent usually has a reaction somewhat more alkaline than strict neutrality (pH = 7·0). The reaction of the medium should then be accurately adjusted.

The apparatus and reagents necessary for adjusting the reaction are:

1. Set of tubes containing solutions of known pH with a definite concentration of indicator.

2. Colour comparator. This consists of a rack to hold the tubes. In its earliest and simplest form it had places for two sets of tubes (Hurwitz, Meyer, and Ostenberg). A later form which is recommended has places for three sets of tubes (Cole and Onslow) (see fig. 2), or a rack may be made to contain a larger series of tubes.

3. Microburette. This may be improvised from an accurate 1·0 c.cm. or 2·0 c.cm. bacteriological pipette divided into 0·01 or 0·02 c.cm. A fine tip is attached to the lower end with rubber tubing and the delivery of the liquid controlled with a pinch-cock.

[1] Coralline (rosolic acid) paper is prepared by dipping thin filter paper or glazed paper into a 0·5 per cent. solution of the indicator in 50 per cent. alcohol. The strips of paper are then dried in the air and stored in a stoppered bottle. In using this paper the earliest change of colour can be detected by allowing paper moistened with the solution to be tested to come into contact with a piece of dry, white filter paper.

4. Bacteriological pipette to deliver 0·5 c.cm. and ordinary pipette of 5·0 c.cm. capacity.

5. Clean cordite tubes.

6. Phenol red solution, 0·01 per cent., made by diluting the 0·02 per cent. solution with an equal volume of distilled water.

7. Twentieth normal sodium hydroxide solution, made by taking 500 c.cm. of 1/10 normal NaOH, 91 c.cm. of 0·01 phenol red, and making up to 1,000 c.cm. with distilled water.

Five cubic centimetres of the medium are measured out into one of the cordite tubes, and 0·5 c.cm. of the 0·01 per cent. solution of phenol red is added. This is put in place 3 in the rack. In places 2 and 6 are put the standard tubes of the reaction between which it is desired to adjust the medium. In place 4 is put a tube of distilled water. In places 1 and 5 are put tubes containing the medium without the addition of indicator.

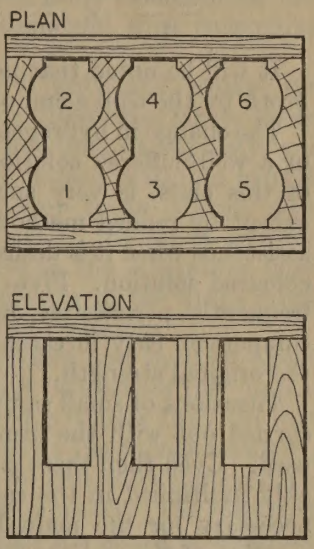

Fig. 2. Colour comparator (3/5 Scale).[1]

In this way the light passing through the standard tubes 2 and 6 traverses tubes containing untreated medium in 1 and 5, while the tube containing medium and indicator in 3 is backed by a layer of water. The conditions under which the light comes through the layers of liquid in all three sets of tubes are thus identical.

Suppose that a reaction of pH = 7·3 is desired. Standard tubes pH 7·2 and pH 7·4 are placed in spaces 2 and 6. It is easy to see from inspection of the standard tubes in the rack whether the medium is more alkaline or more acid than is wished. If the medium is more acid than the standard pH 7·2 it will be necessary to add alkali to tube 3 until the tint of this tube comes between the tints pH 7·2 and pH 7·4. To do this, the 1/20 normal NaOH is added slowly from the micro-burette until the tint is between pH 7·2 and pH 7·4. After the addition of each small quantity of 1/20 normal alkali the contents of tube 3 are well mixed before comparison.[2]

It has been suggested that the 5 c.cm. of medium should be gently boiled for thirty seconds and cooled rapidly before being titrated. This to a certain extent compensates for changes which take place on sterilization, and is, on the whole, a useful procedure.

[1] The comparator may be obtained from Messrs. Baird and Tatlock, Ltd.

[2] For strict accuracy the same quantity of standard alkali should be added at the same rate to tubes 1 and 6, so as to compensate for any colour change of the natural pigment of the medium. Colour changes of this type are especially marked in the case of media made from vegetables.

Knowing the amount of N/20 alkali which is necessary to bring the contents of tube 3 to the required tint, it is easy to calculate how much normal alkali will be necessary to bring a large volume of the medium to the requisite hydrogen ion concentration.

For example:

2,000 c.cm. of a broth are made slightly alkaline to coralline by the addition of normal alkali. A broth is required of pH = 7·4.

In order to bring the tint of a tube containing 5 c.cm. of the medium to a tint which will match the standard 7·4 tube, 0·32 c.cm. of N/20 alkali are required.

Then the amount of normal alkali which will be required to be added to 2,000 c.cm. of broth will be:

$$\frac{2{,}000}{1} \times \frac{1}{20} \times \frac{0{\cdot}32}{1} \times \frac{1}{5} = 64{\cdot}0 \text{ c.cm.}$$

It will be noted that no allowance is made for the dilution of the broth by the 64·0 c.cm. of alkali added.

The change in hydrogen ion concentration which occurs on dilution of a well-buffered solution, and the majority of culture media are of this class, is very small, and is usually incapable of detection except by refined methods. This fact is sometimes of considerable assistance when it is desired to determine the reaction of some highly coloured solution. Five- and even tenfold dilution of a solution is frequently permissible, and this will often render the tintometric comparison easy in cases where it is difficult or even impossible at the original strength.

Titrations of small samples from a large batch of medium must be carried out with the utmost care and accurate apparatus must be used. Even then there is usually a small error, and this is unavoidably multiplied in the calculation of the amount of acid or alkali necessary for the main bulk. In every case, therefore, the final reaction of the end product should be carefully checked.

It should be remembered that media adjusted at room temperature will be considerably more acid at 37° C. At the latter temperature the pH will be 0·3 to 0·4 lower according to the chemical composition of the medium.

It may also be pointed out that media may change in reaction on keeping for a time, owing to the absorption of carbon dioxide from the air. The change is a slow one but may on occasions produce significant results.

Small Scale Methods.

With restriction in the amount of fluid available for experiment accurate determination of reaction becomes increasingly difficult. Drops on test papers give valuable information when large differences are in question, but the method appears to be incapable of refinement and cannot be made to yield precise measurements.

The addition of drops of indicators to drops of fluid on a white surface, such as waxed paper, a glazed tile, or opal glass, gives much

better results. For quick work and preliminary assays it is very useful and with suitable indicators and a good colour memory surprisingly accurate results may be secured.

The method is also capable of being used for fine work, if sufficient care be taken. Felton, who has elaborated it, succeeded in securing a high degree of accuracy by comparing the tint of a drop of fluid to which indicator had been added with the tints of standard mixtures plus indicator, ' spotted ' around the unknown. He recommends the same series of indicators as are advised above for large scale work and also advocates the use of mixtures of these : methyl red with thymol blue for rough work ; methyl red with bromthymol blue ; and methyl red with bromcresol purple. These mixtures give brilliant colour contrasts over a wider zone of hydrogen ion concentration than the individual indicators, and thus economize material.

The method has a good many sources of error and, except for orientation purposes, should be reserved for those cases where other methods are not available. Very minute amounts of impurity will upset the results, e. g. the ash on a freshly flamed platinum loop is often sufficient to alter the reaction of a drop to an entirely new level ; loss of CO_2 from a drop by exposure to the air is another source of error ; traces of ammonia or acid in the laboratory may disturb the results. The proportion of indicator in the drops must be kept constant when small differences are in question.

Other small-scale methods have been devised and most of them have their particular devotees. Mention may be made of the ' Capillator ' put on the market by The British Drug Houses Ltd. In this method, by means of a small rubber bulb and a hard-glass capillary, one volume of appropriate indicator is mixed with one volume of the unknown and the mixture drawn up into the capillary. The resultant colour in the capillary is then matched with the series of accurate standards provided in capillary tubes of the same bore. The range covered by the series of standards extends from pH 2·8 to pH 9·6. It is essential that the capillary tubes be made of glass which does not give off significant amounts of alkali and that the tubes are of the same bore. Owing to the relatively large volume of the indicator solution the method is only applicable to well-buffered solutions. Used with care and judgement the small-scale methods will give valuable results, but they are not to be recommended when adequate supplies are available for the larger scale methods.

Increase in Hydrogen Ion Content on Sterilization.

All bacteriologists have noted that media become more acid on sterilization. The difficulty in ensuring that a medium shall have a definite hydrogen ion concentration after sterilization is great. It is increased when, as is often the case, the medium contains glucose or lactose, and when the medium is sterilized in tubes.

If an excess of alkali be added to a medium containing glucose

or lactose, it is rarely found that the reaction of the medium is alkaline after sterilization. More often the hydrogen ion concentration rises to pH = 6·0. The attempt to compensate for this by adding an excess of alkali before sterilization is ineffective, because, owing to the increase of hydroxyl ions, the acid products are produced in greater quantity from the sugar. The products formed by the action of alkalis on sugars are many, and the acids which may be formed vary from saccharic acid to lactic acid. Another feature of the action of sugars in media containing amino acids is that these two combine to form so-called humin substances. To this is due the darkening on sterilization of peptone solutions containing glucose. One never obtains a sterile glucose peptone solution so light in tint as a plain broth containing no carbohydrate.

The only certain method of obtaining a glucose or lactose medium with a pH greater than 7·0 is to add sterile sugar solution after sterilization of the nitrogenous part of the medium.

In this way the technical difficulties of preparing carbohydrate-containing media of a pH greater than 7·0 are increased. With careful asepsis in a still room sterile sugar solutions may be added to broth with the loss of comparatively few tubes. The methods for doing so are given in any text-book on bacteriology.

Reaction of Solid Media.

Gelatine. The reaction of media containing gelatine may be easily determined if the medium is liquefied at 30° C. As a rule, gelatine is distinctly acid in reaction, and its addition to broth causes a rise in the hydrogen ion concentration of the mixture.

Agar. The addition of a good quality agar to broth has but little effect on the latter. The reaction of the medium may be estimated in the liquefied medium, but the temperature at which this is done makes the determination difficult. It is therefore better to use a clean agar which, preferably, has been washed, and take the reaction of the broth as that of the complete medium.

The reaction of the finished medium may be checked by adding the indicator to the liquefied medium, mixing it well and allowing it to cool thoroughly. The colours may then be compared, using the same precautions as in the case of broth.

It is sometimes impossible to secure good colour matches with solid media, the tint of the indicator being altered by the physical state of the medium, and checking the reaction of the finished product is then far from easy. The difficulty is best met by free dilution. Agar or gelatine should be diluted ten times with boiling water and then cooled. At this dilution there is a time interval in which the medium remains fluid and accurate tintometry is quite easy. This method is recommended by Culyer, who has employed it as a routine.

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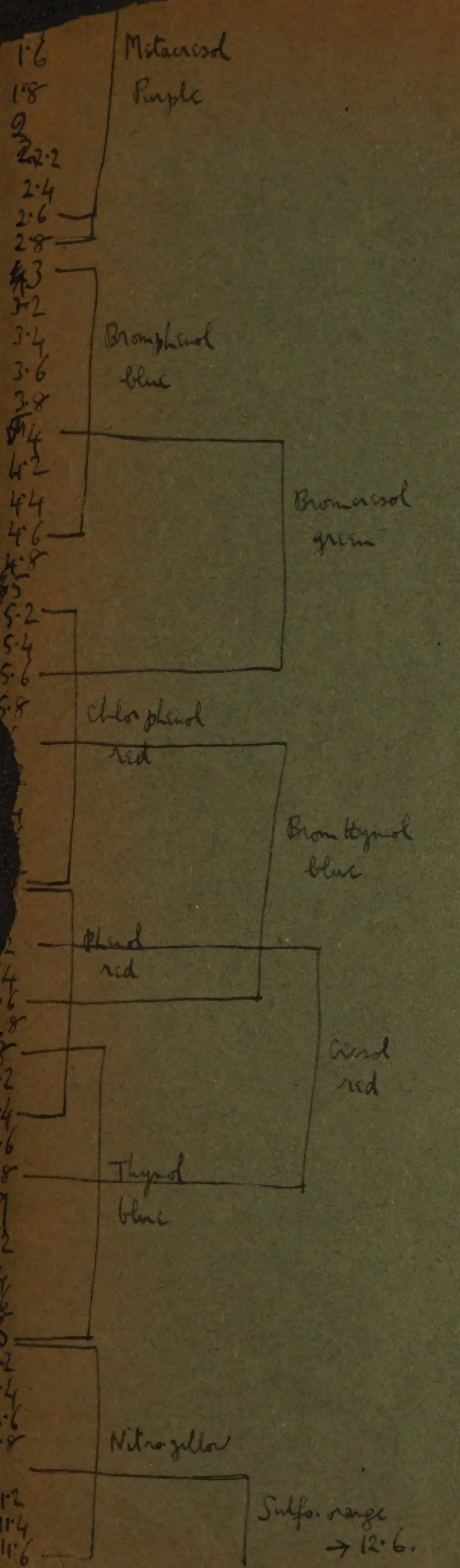
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